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VALIDATED CHROMATOGRAPHIC METHODS FOR STANDARDIZATION OF RESTOBAL LIQUID – AN IMMUNOMODULATORY AND ANTI STRESS POLYHERBAL PRODUCT

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ABSTRACT

Standardization is the code of conduct in order to ensure the efficacy that manufacturers should use to ensure batch to batch consistency of the product. Efficient standardization and quality control of multi-herbal formulations can be achieved by adopting validated methods on the sophisticated instrument like HPLC and HPTLC. Standardization of the product Restobal liquid with respect to its phytotherapeutic constituents was undertaken wherein HPLC and HPTLC methods for Glycyrrhizin and Gallic acid estimation respectively were optimized and validated using the linearity, selectivity, precision, recovery, LOD & LOQ parameters in accordance with the statistical method of validation given in *ICHQ2R1*. The average recovery of (Glycyrrhizin) 98.68 % and (Gallic acid) 99.99 % were computed from the regression equation. RSD for inter-day and intra-day variability was also found to be less than 1%. The method thus developed is being successfully applied in identification and quantification of phytotherapeutic constituents.

KEYWORDS: Standardization, Glycyrrhizin, Gallic acid, Restobal, HPLC, HPTLC.

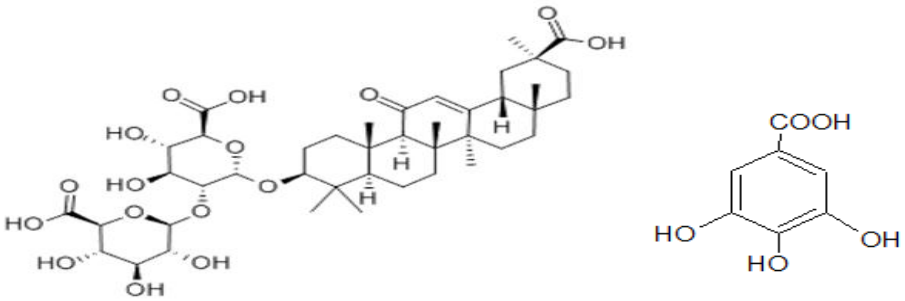
1. INTRODUCTION

Unique bio marker compounds as active constituents present in the herbs ensure the efficacy of the polyherbal formulation. To quantify active ingredients in the complex polyherbal composition the development of authentic analytical methods is a major challenge to scientists. Standardization with respect to the active biomarker compounds ensures the batch to batch consistency of the product.[1-3]

Stress is defined as "the physical pressure, pull, or other force exerted on one thing by another; strain," or "a specific response by the body to stimulus, such as fear or pain, that disturbs or interferes normal body physiological equilibrium." Stress can be considered anything that is applied to an animal from an outside source that has an effect on that animal's "normal" physiological activity. Oxidative stress is an imbalance between the systemic manifestation of reactive oxygen species and a biological system's ability to readily detoxify the reactive intermediates or to repair the resulting damage.[4] Stress evokes harmful responses that interferes with the general health, productivity and result in immunosuppression.

Restobal, a proprietary polyherbal formulation of AYURVET for cattle is a complete potent herbal antistress, adaptogenic, restorative, immunomodulator and performance enhancer. Its constituent herbs namely *Glycyrrhiza glabra, Phyllanthus emblica, Shilajit, Asparagus racemosus, Mangifera indica, Tribullus terrestris, Withania somnifera* and *Ocimum sanctum* have been scientifically well established for antistress, adaptogenic, immunostimulant, antioxidant and free radical scavenging activities.[5-7]

Standardization of the product with respect to the bioactive phyto constituents was taken up to insure the batch to batch consistency in efficacy. New HPTLC and HPLC methods were developed for the quantification of two main ingredients of the formulation i.e. *Glycyrrhiza glabra* with respect to bioactive saponins Glycyrrhizin (Fig. 1, A) having free radical scavenging and Anti-oxidant activities and *Phyllanthus emblica* with phenolic bioactive compound Gallic acid (Fig. 1, B) having antioxidant and immunomodulatory activity. The analytical methods were validated as per ICHQ2R1 guidelines.[8] Limits of the biomarker were set as check point for the formulation efficacy.



(A) **(B)**

Fig 1: Structure of Glycyrrhizin (A) and Gallic acid (B).

2. EXPERIMENTAL

2.1 Apparatus

HPTLC was performed with Camag HPTLC equipment (Muttenz, Switzerland) comprising Linomat V auto sample applicator, Camag Scanner-III, Camag flat bottom and twin trough developing chamber, and UV cabinet with dual wavelength UV lamp. 20 × 10 cm aluminum 60F254 TLC plates (E-Merck-Germany) with stationary phase silica gel and layer thickness 0.2 mm were used for the resolution of chemical constituents. HPLC was performed with WATERS, USA having binary pump 515 with PDA 2996 detector. The data was acquired on the Empower 2.0 controlling software. Separation was obtained on Phenomenex luna C18 column (250 mm x 4.6 mm, 5 µm).

2.2 Reagents and materials

Chemicals and reagents used were of analytical reagent grade. Toluene, Ethyl acetate, Formic acid, Chloroform, Methanol and Water were purchased from RANKEM. Gallic acid was purchased from HIMEDIA and Glycyrrhizin was isolated in our lab and structure was established by interpreting the 1H, 13C and 2D NMR spectra. Potassium dihydrogen orthophosphate-AR grade was purchased from SD Fine-Chem limited. TLC plates were purchased from Merck (Darmstandt, Germany). Controlled samples of Restobal liquid were obtained from the QA/QC department of AYURVET LTD, Baddi.

2.3 Chromatographic conditions

HPTLC was performed using commercially-prepared, pre-activated (110°C) silica gel 60 F254 TLC plates. A Linomat V (Camag, Muttenz, Switzerland) automatic TLC applicator was used to apply samples and standards (marker compounds) on pre-activated (110°C) silica gel 60 F254 TLC plates under a flow of nitrogen gas and the delivery speed of the syringe was 10 s/ µl. Each TLC plate was developed to a height of about 9.0 cm, under laboratory conditions. Toluene : Ethyl acetate : Formic acid : Water (3:3:0.8:0.4 v/v/v/v) was the mobile phase developed for the resolution of and quantification of Gallic acid (Fig1. B). Quantitative determination of gallic acid spots were done by Camag TLC Scanner 3 at 280 using deuterium lamp with a slit size of 6 × 0.3 mm.
HPLC was performed with WATERS, USA having binary pump 515 with PDA 2996 detector. The data was acquired on the Empower 2.0 controlling software. Separation was obtained on Phenomenex luna C18 column (250 mm x 4.6 mm, 5 µm). The mobile phase was filtered through 0.45 µm Millipore filter and degassed. To optimize the RP-HPLC parameters, several mobile phase compositions were tried. A satisfactory separation and good peak symmetry for Glycyrrhizin (Fig1. A) was obtained with a mobile phase, buffer solution of (5.3 mmol) Potassium dihydrogen orthophosphate solution : Acetonitrile :: 65 : 35 of pH 3.0 adjusted with acetic acid. Flow rate of 0.7ml/min was set to get better reproducibility and repeatability. Selecting 254.0 nm as the detection wavelength resulting in acceptable responses and enabled the detection of compound under investigation.

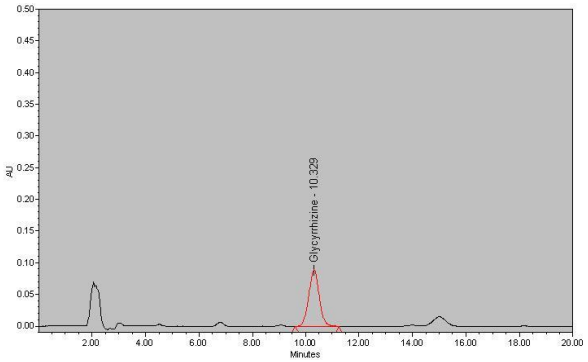
2.4 Preparation of sample and standard solutions

2.4.1 Preparation of standard solutions

Stock solutions (0.1 mg/ml) of standards (marker compounds) A and B were prepared in methanol, different concentrations were spotted/injected in order to prepare the calibration graphs and quantification of bioactives.

2.4.2 Preparation of sample solution for gallic acid estimation: Weighed accurately around 2.0 g of Restobal liquid and transferred to a 50 ml round bottom flask. Added 30 ml of methanol sonicated for 15 minutes and made up the volume to 50ml. Filtered the solution through 0.45µ syringe filter before application of spots on TLC.

2.4.3 Preparation of sample solution for quantification of Glycyrrhizin: Sonicated 5.0 g of Restobal liquid in a 100 ml volumetric flask with 75 ml of methanol for 15 minutes, adjusted the volume to the mark with same solvent. Filtered the solution through 0.45 µ filter before injecting into HPLC.



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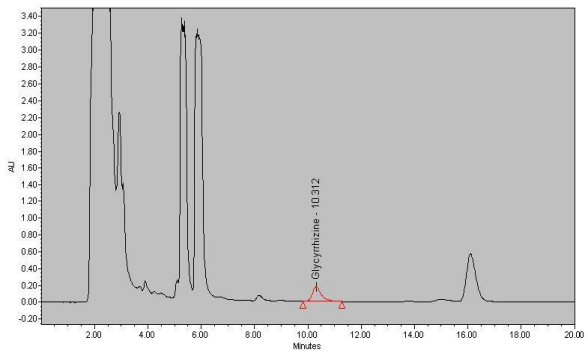

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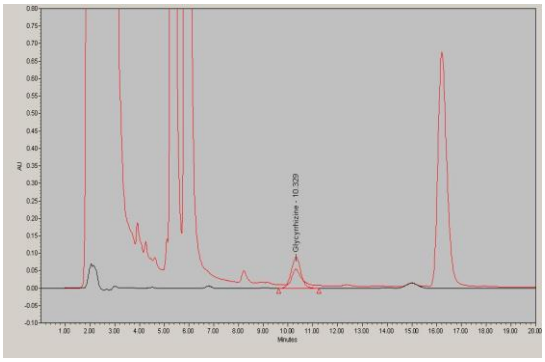

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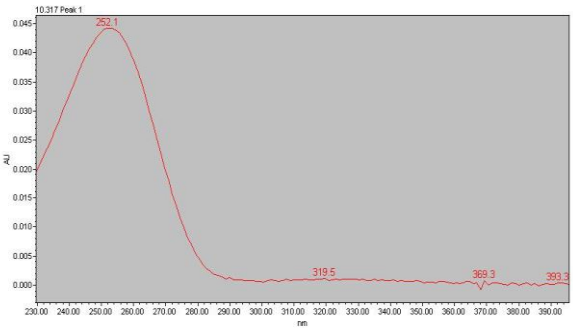

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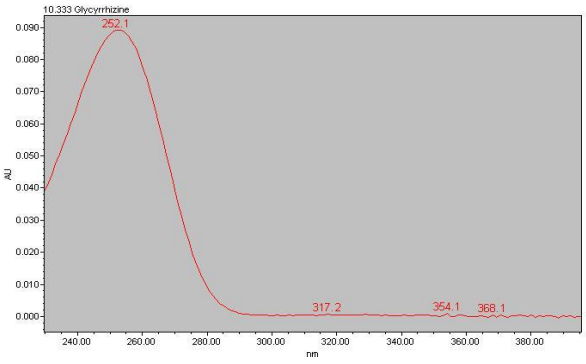

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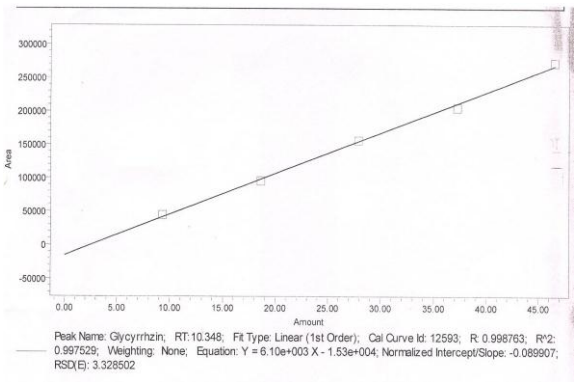
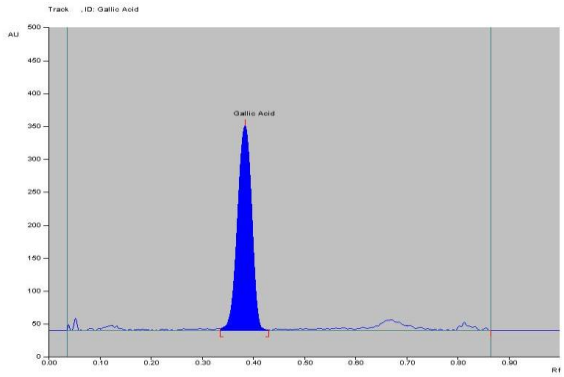


(f)

Fig 2: Chromatograms showing the resolution of the marker compounds in the formulation Restobal liquid. (a) Chromatogram of marker compound Glycyrrhizin (A). (b) Chromatogram of the formulation Restobal liquid. (c) Overlay of chromatograms of Glycyrrhizin standard with its counterpart in formulation. (d) Spectral chromatogram of Glycyrrhizin standard. (e) Spectral chromatogram of the Glycyrrhizin in formulation Restobal liquid. (f) Calibration plot for Glycyrrhizin standard.



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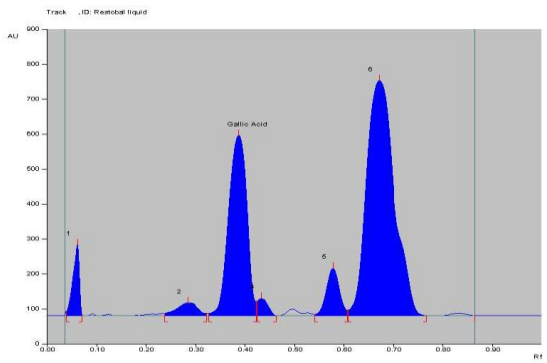

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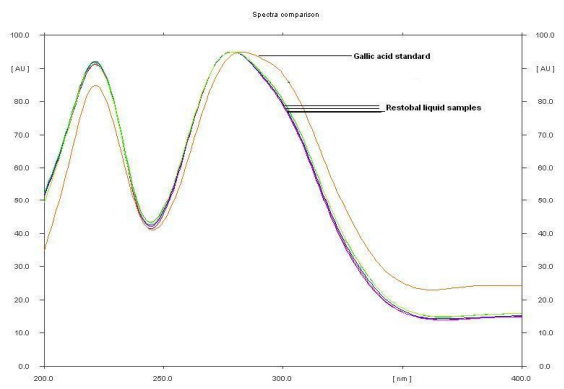

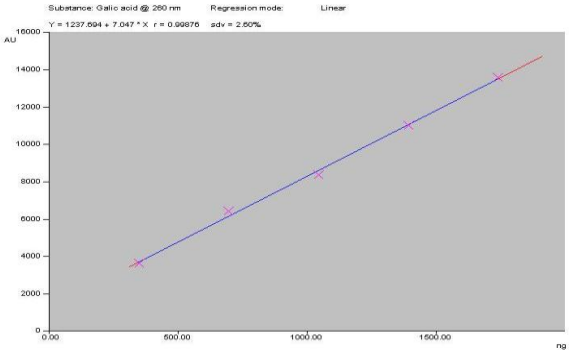

(c) **(d)**

Fig 3: Chromatograms showing the resolution of the marker compounds in the formulation Restobal liquid. (a) Chromatogram of marker compound Gallic acid (B). (b) Chromatogram of the formulation Restobal liquid. (c) Overlay of Spectral chromatograms of Gallic acid standard with its counterpart in formulation Restobal liquid. (d) Calibration plot for Gallic acid standard.

Table 1. Results of precision, linear regression analysis and their correlation coefficient for quantitative analysis of different marker compounds.

Parameters	Gallic acid	Glycyrrhizin
Concentration range [µg/ng]	0.5 – 2.5 µg/spot	09 - 45 µg/ml
Regression equation	y = 7.047x + 1237.69	y = 6.10 x – 1.53
Correlation Coefficient (r2)	0.998	0.997
Amount of marker compound in Restobal liquid [%w/w] [a]	0.150 %w/w	0.024 %w/w
Method precision (Repeatability) – RSD %	0.95	0.91
Intermediate precision (Reproducibility) - RSD [%] Intraday 1 Interday 3	 0.99 0.91	 0.86 0.93
LOD	0.02 µg $spot^{-1}$	0.24 µg ml^{-1}
LOQ	0.06 µg $spot^{-1}$	0.72 µg ml^{-1}

y = peak area response
x = amount of marker compound
[a] = Mean, n=5

Table 2: Results from determination of recovery.

Parameter	Gallic acid			Glycyrrhizin		
Initial concentration in formulation [mg g-1]	1.50	1.50	1.50	0.24	0.24	0.24
Concentration added [mg g-1]	0	8.0	16.0	0	2.0	4.0
Total concentration [mg g-1]	1.50	9.50	17.5	0.24	2.24	4.24
Concentration found [mg g-1]	1.51	9.49	17.4	0.23	2.25	4.23
RSD [%] (n=7)	0.96	0.89	0.99	0.90	0.96	0.98
Recovery [%]	100.67	99.89	99.42	95.83	100.45	99.76
Mean recovery [%]	99.99			98.68		

3. RESULTS AND DISCUSSION

The exercise was carried out to ensure the consistency in the desired pharmacological effect by establishing the lowest possible limit for two of its most relevant bioactive phytoconstituents. Experimental studies have already been carried out to evaluate antistress activity of *Glycyrrhiza glabra* and *Phyllanthus emblica.* Standardization of these phytotherapeutic constituents with validated analysis methods will ensure the batch to batch consistency in efficacy of the product on commercial scale.

As two herbs mentioned under experimental investigation are among the main active ingredients in the polyherbal formulation, quantifying them with their respective bioactive markers and setting the limits will help us in ensuring authenticity and efficacy of the product in turn.

3.1 METHOD VALIDATION

Validation parameters: The methods were validated according to ICH guidelines for linearity, precision, accuracy, selectivity, limit of detection and limit of quantification.

3.1.1 Calibration curve (Linearity)
The method was validated in accordance with the statistical method of validation given in ICHQ2R1 (5). Two independent calibration equations were obtained. Linear regression analysis was used to calculate the slope, intercept, and coefficient of determination/regression coefficient (r2) for each calibration plot. Responses were linear in the concentration ranges investigated. Quantification was on the basis of peak area.

3.1.2 Calibration: The marker compounds in the formulation were quantified using calibration curves established with five dilutions of the standard at concentrations ranging from 0.5 – 2.5 µg/spot for the Gallic acid and 09 - 45 µg/ml for Glycyrrhizin standard compounds (Table 1; Figures 3d and 2f). The corresponding peak area in the formulation was plotted against the concentrations of the standard injected. Peak identification was achieved by comparison of both the Rf/RT and UV absorption spectrum with those obtained for standard.

3.1.3 Linearity: Linear regression analysis was used to calculate the slope, intercept, and coefficient of determination/regression coefficient (r2) for calibration plot. Linearity was determined by using five concentration of the standard solution. The calibration curve was obtained by plotting the area versus the concentrations of the standard solution. Responses were linear in the concentration ranges investigated (Table 1; Figures 2f and 3d)

3.1.4 Range: Range is the interval between upper and the lower concentration (amount) of analyte in sample for which it has been demonstrated that the analytical method has suitable level of precision, accuracy and linearity. The linear response were observed at 280 nm / 254 nm over a range of 0.5 – 5.0 µg/spot for the Gallic acid and 09 - 45 µg/ml for Glycyrrhizin standard compound.

3.1.5 Accuracy (% Recovery)
Recovery experiments were conducted to check for the presence of positive or negative interferences from other ingredients/excipients present in the formulation and to study the accuracy of the method. Recovery was determined by the standard addition method. Gallic acid & Glycyrrhizin standards were added to the formulation at two different concentrations, extraction and analysis was performed as described in preparation of sample solution. Recovery was calculated for each standard at each concentration. The results obtained are listed in Table 2.

3.1.6 Precision
Three different concentration of the marker compound solution in triplicates were injected on three different times within the same day and repeating the same on three different days to record intra-day and inter-day variation in the results. The low %RSD values of intraday and inter day for marker compound reveals that the proposed method is precise (Table 1.).

3.1.7 Selectivity
The selectivity of the respective method was determined by comparing the retention factor and absorbance spectrum of the standards and the corresponding peaks obtained from the extracts of the formulation. The UV-Vis spectra of both the compounds were compared at three different positions, the peak start, peak center, and peak end. There was good correlation between spectra obtained at each of the three positions. The Glycyrrhizin & Gallic acid peaks separately were, therefore, not masked by any peak of other compound present in the formulation (Figures 2 c,d,e and 3 c), which indicated respective peak purity.

3.1.8 LOD & LOQ
The LOD, defined as the amount of compound required to produce a signal at least three times the noise level. The LOQ, defined as the amount of compound required to produce a signal at least ten times the noise level. The LOD for Gallic acid & Glycyrrhizin was 20 ng $spot^{-1}$ and 0.24 µg ml^{-1} respectively, whereas, the LOQ was 60.0 ng $spot^{-1}$ and 0.72 µg ml^{-1} respectively.

Quantification of Glycyrrhizin & Gallic acid in clinically efficacious batches helped in standardization of the product with respect to its phytotherapeutic constituents. The newly developed method ensures the batch to batch consistency in efficacy of the product on commercial scale.

4. CONCLUSION
New HPLC and HPTLC methods were developed for the fine resolution of various phyto constituents of the product. Standardization of phototherapeutic constituents Glycyrrhizin and Gallic acid possessing strong free radical scavenging and antistress activities with validated analysis methods helped and will help in ensuring the batch to batch consistency in quality & efficacy of the product on commercial scale.

5. ACKNOWLEDGEMENT
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